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14. ABSTRACT
Short CUX1 isoforms were found to be overexpressed in breast cancer cell lines, in human breast tumors and in uterine leiomyomas, suggesting that these proteins play a key role in tumor development and progression. My project consists in analyzing the effect of these CUX1 isoforms on mammary gland development and tumorigenesis. Also, I will work on the identification of targets of CUX1 that mediate its oncogenic properties. So far, I have shown that overexpressing short CUX1 isoforms leads to abnormal development of the mammary gland. Furthermore, overexpressing p75, p110 or p200 CUX1 leads to the development of mammary gland tumors in mice. These tumors seem to be of basal origin, suggesting that CUX1 promotes tumorigenesis in a precursor cell. Breast tumor patients with similar types of disease have very low chances of survival, since no specific treatment is currently available for them. Thus, my research project will enable us to gain a better understanding of the biological functions of each CUX1 isoform in mammary gland development and tumorigenesis, which could possibly lead to new therapeutic targets for the treatment of basal breast cancer.

15. SUBJECT TERMS
Cancer, Breast Cancer, Oncogene, Proliferation, CDP/Cux, CUX1, Transcription factor

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Table of Contents

INTRODUCTION

The CUX1 transcription factor (CUX1 is the new nomenclature for CDP/Cux) is an important regulator of cell cycle progression, and was also found to be involved in many other processes, such as determination of cell-type identity, cell growth control, as well as cell migration and invasion (*1-4*). In addition, short CUX1 isoforms were found to be overexpressed in breast cancer cell lines, in human breast tumors and in uterine leiomyomas, suggesting that these proteins could play a key role in tumor development and progression (*5, 6*). I have also previously shown that overexpression of p75 CUX1 results in the development of myeloproliferative disease-like myeloid leukemia in mice (*7*). My project consists in analyzing the effects of these CUX1 isoforms on mammary gland development and tumorigenesis, which will allow us to demonstrate the oncogenic role of CUX1 on mammary epithelial cells. I will also work on the identification of targets of CUX1, the deregulation of which could lead to cancer progression. My research project will enable us to gain a better understanding of the biological functions of each CUX1 isoform in mammary gland development and tumorigenesis. Any knowledge gained in this area is very important, as it could lead to new therapeutic targets for the treatment of breast cancer. Briefly, transgenic mice overexpressing p75, p110 or p200 CUX1 under the control of the mouse mammary tumor virus promoter (MMTV) specifically integrated into the hypoxanthine phosphorybosyltransferase (hprt) locus were generated. These mice were found to display anomalies in mammary gland development and to develop tumors, which is what I will describe in this report. This study mainly examines mammary gland tumor development in CUX1 transgenic mice of pure FVB background.

BODY

1-Determine the effect of the overexpression of CUX1 p200, p110 and p75 isoforms on mammary gland development (Task 1)

Mammary gland wholemounts and H&E stainings from mice from the different lines were examined at different stages of development including 5 weeks, 3 months, 6 months, pregnant, lactating and involuting. Mammary glands of transgenic mice were normal at all stages, except for virgin homozygotes p75 CUX1 at 5 weeks and virgin homozygotes p200 CUX1 at 3 months. First of all, homozygous p75 mammary glands had a longer ductal outgrowth than wild-type mammary glands. As can be seen in Appendix 1A, the ducts in p75 mice have reached far beyond the lymph node, while wild-type ducts have just reached the lymph node at this time point. However, this difference is lost in further time points since wild-type ducts fill out the whole mammary gland equally well. For instance, at three months of age, there is no more difference between wild-types and p75 mammary glands (Appendix 1B). The second difference observed is an increased branching with increased number of end buds in p200 homozygotes at 3 months of age (Appendix 1B). To determine if this difference was due to increased proliferation, immunohistochemistry was performed using PCNA as a proliferation marker on wild-type versus p200 CUX1 mammary glands (Appendix 1D). PCNA stainings seemed to be similar in both samples. Other stainings should be performed at earlier time points to rule out a difference in proliferation. No differences were seen for the p110 mice at any time point. The slight increase in branching in p110 CUX1 mice observed at 3 months of age (that was reported in annual summary 2007) was found to be normal and to be due to estrous cycle variations.

These results suggest that overexpressing CUX1 in the mammary gland results in very small differences in mammary gland development. This is probably due to the very low levels of expression of transgenic CUX1 isoforms at these stages, as seen in Appendix 1C. The fact that the different transgenic mouse lines have different phenotypes in this developmental study suggests that each CUX1 isoform potentially has a different function in mammary gland development.

2-Determine the effect of overexpressing CUX1 p200, p110 and p75 isoforms on mammary gland tumorigenesis in mice having reached a pure genetic background (Task 2)

Transgenic mice overexpressing p75, p110 or p200 CUX1 develop mammary gland tumors

Heterozygous FVB mice overexpressing p75 CUX1 (n=30), p110 (n=87) or p200 (n=51), as well as wild-type mice (n=74) were bred multiple times to stimulate transgene expression and were left to age until moribund or until 24 months of age. Appendix 2 shows a table with the percentages and types of tumors developed in the various lines. Overall, mice from the 3 lines develop more tumors of all types combined than wild-type mice, with percentages varying from 40 to 50 as compared to 19% in wild-type mice. For the three lines, this increase is statistically significant. The transgenic mice develop mainly more mammary gland and lung tumors. Our study will focus on the mammary gland tumors. Whereas 4% of wild-type mice develop mammary gland tumors, 10 to 20% of transgenic mice develop such tumors. These mice developed tumors with long latencies, ranging from 17 to 24 months, which suggests that CUX1 acts in collaboration with other events to promote tumorigenesis. We have generated groups of homozygous mice to increase transgene expression and potentially decrease latency. These mice are currently being monitored for tumor development.

Tumors display various histologies

Tumors of p75, p110 or p200 origin were found to have very different histologies by H&E staining analysis (Appendix 3A). Some are adenosquamous carcinomas, some are solid carcinomas and others are adenomyoepithelioma. Some tumors also contain different histological types within the same mass. A summary of these differences can be seen in a table format (Appendix 3B). This analysis reveals that mice from the three different lines develop different types of tumors, but mainly adenosquamous and solid lesions. To confirm heterogeneity of tumor types, cytokeratin stainings were performed (Appendix 3C). CK6 stains progenitor cells, CK14 stains myoepithelial cells and CK8/18 stains luminal epithelial cells. Consistent with this heterogeneity, some tumors are CK6+,CK14+,CK8/18+ (less differentiated tumors), others are CK6-CK14+CK8/18+ and finally some are CK6-CK14-CK8/18+ (more differentiated tumors). These results suggest that CUX1 targets malignant transformation of a precursor cell, which can then give rise to different lineages. This suggests that the tumors developed in those mice are basal (*8-12*). Such a conclusion, however, can only be confirmed by expression profiling analysis on the tumors, which will be performed in our lab.

The transgene is expressed and active in mammary gland tumors

RNA extracts were prepared from mammary gland tumors and from the adjacent normal mammary gland of the same mouse (Appendix 4A). cDNA was prepared and was used to specifically amplify the transgene by PCR. From this analysis, we can see that there is consistently more expression of the transgene in the tumor than in the adjacent mammary gland tissue, with often no detectable level of expression of the transgene in the latter. Immunohistochemistry was performed using the 1300 CUX1 antibody and allowed us to detect transgene expression at the protein level in the tumors (Appendix 4B). By western blot analysis using the 1300 CUX1 antibody, we can see that the transgene expression is increased in the tumors versus adjacent tissue (Appendix 4C). These results strongly suggest that the tumors are caused by the overexpression of CUX1. Southwestern analysis was performed to look at DNA binding activity of CUX1 in the tumors. This technique involves renaturation of the protein on a membrane and incubation of the renatured protein in the presence of radiolabeled DNA probes containing the binding sequence of CUX1. In all cases analyzed, CUX1 binding was increased in tumors versus adjacent, suggesting that the CUX1 transgene is active in the tumors.

In p200 tumors, there is more p110 and it is active

Whereas a role for short CUX1 isoforms is being more and more confirmed, a role for p200 in oncogenicity has remained more elusive. Therefore it was surprising to see that p200 mice developed as many tumors as p110 mice. One possible explanation for this is that perhaps more short isoforms are generated from the p200 protein due to increased proteolytic processing. First of all, we can see that the p200 tumors express the transgene at the mRNA level (Appendix5A) and at the protein level (Appendix5B). Moreover, as predicted, we can see that p200 tumors express more p110 and that this p110 is active as detected by southwestern analysis (Appendix 5B). Thus, these results suggest that tumorigenesis possibly occurs through overexpression and increased activity of p110.

Increased expression of Cathepsin L in p200 tumors, but not in p110 or p75 tumors

A possible explanation for the generation of more p110 from the p200 tumors is increased proteolytic cleavage by Cathepsin L. cDNA was prepared from tumor material from the different lines and expression of Cathepsin L was quantified by Real-Time PCR analysis. Interestingly, most p200 tumors contained more Cathepsin L expression whereas most p110 and p75 tumors did not (Appendix 6A). Thus, overexpression of Cathepsin L could be a collaborating event required prior to or during tumor formation in p200 transgenic mice.

Activating ras mutation in one p200 tumor

It was reported previously that Cathepsin L expression is increased downstream of ras activation (*13-17*). Therefore, we decided to look at ras-activating mutations in p200 tumors. Out of three tumors analyzed so far, one had indeed a ras-activating mutation (tumor 2, Appendix 6B). Other members of the ras-signaling cascade, such as raf, will also be looked at, and more p200 tumors will be analyzed. This analysis will help determine if activation of the ras-signaling cascade is a preferred pathway leading to mammary gland tumorigenesis in p200 CUX1 transgenic mice.

Some tumors have the potential to metastasize to distant sites

Most tumor-bearing mice did not develop metastasis to distant sites. However, one p75 mouse (Appendix 7A) and one p200 mouse (Appendix 7B) did develop metastases to the lungs. Therefore, metastasis sometimes occurs in CUX1 transgenic mice, but it is a rare event. Thus it is probable that additional collaborating events are needed for the CUX1 tumors to become metastastic.

Tumor-derived cell lines still express the transgene and have different morphologies

Cells lines were established from the mammary gland tumors and it was found that these cell lines have varying morphologies. Some cell lines are spindle-like and others are more epithelial-like. Finally, others have two nuclei per cell, suggesting aneuploidy (Appendix 8A). These different morphologies fit very well with the fact that the original tumors have various histologies. Additionally, the cell lines derived from the mammary gland tumors were found to maintain expression of the transgene (Appendix 8B, western blot, lane 1(scramble)).

When transgenic CUX1 is knocked-down in tumor-derived cell lines, these cells have reduced migration capacities

DicerRNA was used against CUX1 in tumor-derived cell lines to knock down exogenous CUX1. After proper verification of CUX1 knockdown, a migration assay was performed using transwells. This analysis revealed that knocking down CUX1 in tumor-derived cell lines seriously affected the cells' capacity to migrate, since motility was significantly reduced in dicer-treated cells in both a p110 cell line (Appendix 8B) and two p200 cell lines (Appendix 8C and D). This assay revealed that CUX1 is necessary not only to initiate cancer development in these mice, but also to help the migration of the cells. Using a cysteine protease inhibitor that inhibits Cathepsin L in a p200-derived cell line in order to block processing of p200 into p110, we also showed that blocking cleavage of p200 into p110 results in reduced migration capacities (Appendix 8E).

Wnt pathway is altered in tumors

Since deregulation of the β-catenin/wnt pathway generates similar types of mammary gland tumors in mouse models (*9*), we decided to look at this pathway in the CUX1 tumors. First of all, ChIP-on-Chip analysis revealed that multiple Wnt genes are putative transcriptional targets of CUX1. Therefore, we decided to look at expression of these wnt genes in the tumors. As seen in Appendix 9, many tumors display overexpression of Wnt genes, particularly of Wnt1 (8 out of 11 tumors overexpress Wnt1) and Wnt10A (7 out of 9 tumors). Future work should focus on validating that these wnt genes are true targets of CUX1 by Chromatin immunoprecipitation (ChIP), and analyzing the effect of such overexpression on the wnt/β-catenin pathway through the study of β-catenin localization and expression at the protein level in tumors by immunohistochemistry. The expression of Her2 (erbB2) was also looked at in a few tumors and its expression was found increased in 3 out of 4 tumors. This increase means that Her2 could be a collaborating factor in the onset of mammary gland lesions in CUX1 mice. Expression of Her2 in more tumors will be looked at as well as its protein expression. Other genes that have been identified as putative targets of CUX1 by ChIP-on-Chip and that I will test in mammary gland tumors include Brca1 and Tsg101.

3-Generate bigenic mice overexpressing CUX1 and expressing activated ErbB-2 to study collaboration between the two oncogenes (months 18-36)

Breedings are under way.

KEY RESEARCH ACCOMPLISHMENTS

-Some developmental anomalies are observed in mice overexpressing CUX1:
- -faster ductal outgrowth in p75 CUX1 mammary glands at 5 weeks of age
- -increase in ductal branching in p200 CUX1 mammary glands at 3 months of age

-Overexpressing any of the three CUX1 isoforms p75, p110 or p200 in mammary gland epithelial cells result in tumor development. Thus, CUX1 is an oncogene.

-Tumors developed in the CUX1 transgenic mice are heterogeneous, which suggests that CUX1 possibly could be involved in specifying a basal phenotype.

-p200 CUX1 tumors display enhanced p110 binding to DNA, suggesting that tumors formed in this mouse line are due to increased proteolytic processing of p200 into p110, yielding more of the short oncogenic CUX1 isoform.

-Some CUX1 tumors metastasize to the lungs.

-wnt1 and wnt10a are overexpressed in many mammary gland tumors, suggesting that the wnt/β-catenin pathway could be activated in the CUX1 tumors.

REPORTABLE OUTCOMES

Manuscript in preparation

Overexpressing Short CUX1 Isoforms p75 and p110 in Transgenic Mice Leads to Mammary Gland Tumor Development
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Molecular Oncology Group, McGill University Health Center[1], Departments of Biochemistry[2], Oncology [3] and Medicine[4], McGill University, Montreal, Canada.

Abstracts

- Chantal Cadieux[1,2], Laura Hulea,[1,2], Alain Nepveu[1,2,3,4]
CDP/Cux Transgenic Mice: The role of CDP/Cux in Cancer
Mechanisms and Models of Cancer Meeting, Salk Institute, La Jolla, August 8-12, 2007.

Oral Presentations

The role of CUX1 in Cancer
Molecular Oncology Group Talk, McGill University, Montreal, January 8, 2008

CONCLUSION

Through this project I will assess the role of different CUX1 isoforms on the development of the mammary gland as well as on tumor development. So far, I have shown that overexpressing p75, p110 or p200 CUX1 leads to anomalies in mammary gland development and also leads to tumor formation. I have also shown that CUX1 tumors seem to be of basal origin since they are very heterogeneous histologically and express cytokines associated with different lineages, namely cytokeratin 6 (precursor cells), cytokeratin 14 (myoepithelial cells) and cytokeratin 8/18 (luminal epithelial cells). Furthermore, some of these tumors have the potential to metastasize to the lungs. Tumors originating from p75 or p110 CUX1 mouse lines display enhanced expression of the transgene and the transgene was shown to be active by southwestern analysis. In addition, p200 CUX1 tumors were also found to overexpress short isoforms of CUX1 and to display increased DNA binding activity of p110. This increase in p110 expression could be due to increased expression of the cysteine protease Cathepsin L since p200 tumors overexpress this protease whereas tumors formed in the p75 and p110 lines do not. By looking at expression of Wnt genes in the CUX1 tumors, I found that Wnt1 and Wnt10a were overexpressed in many tumors.

In the future, I plan to study the wnt/β-catenin pathway in more details, since mice in which this pathway is deregulated develop similar tumors. I also plan to study the ras pathway in the p200 tumors to decipher the mechanisms leading to the overexpression of Cathepsin L in the p200 tumors. In regards of this, I will further work with p200 mammary gland tumor-derived cell lines and test Cathepsin L inhibitors and their impact on cell proliferation, migration and invasion. I will also look at more putative targets of CUX1, such as Brca1 and tsg101 that could play a role in mammary tumorigenesis.

“So what section”

My research has identified a new oncogene. I have provided evidence that overexpressing CUX1 contributes to the malignant transformation of epithelial cells in the mammary gland and thereby causes cancer in mice. This will allow identification of new targets for therapeutic drug development against breast cancer. Also, in my future work, I plan to identify some targets of CUX1 that mediate part of this oncogenic phenotype, which will further help identify more targets for possible therapeutic drugs. Futhermore, that discovery that Cathepsin L is increased in the p200 tumors has confirmed that this protease could be a target for breast cancer treatment. Inhibitors of Cathepsin L already exist and will be further studied in this research project.

Furthermore, my research has enlightened the fact that overexpressing CUX1 seems to be associated with the development of tumors in mice that resemble a specific category of breast tumors called basal tumors in humans. These breast cancers are much harder to treat and often recur because we lack specific molecules to target to kill these cancer cells. My research will possibly help identify one of these targets.

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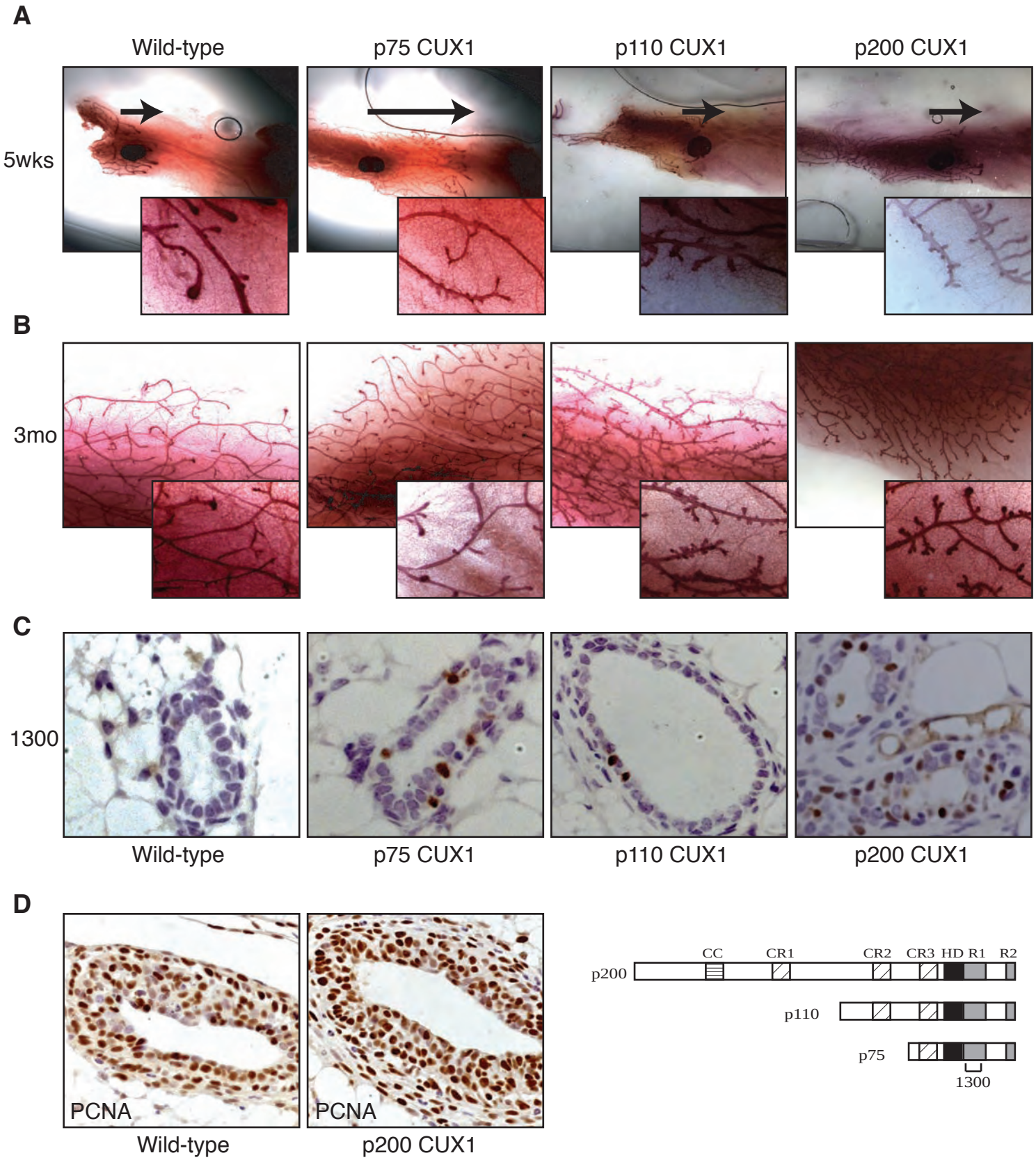
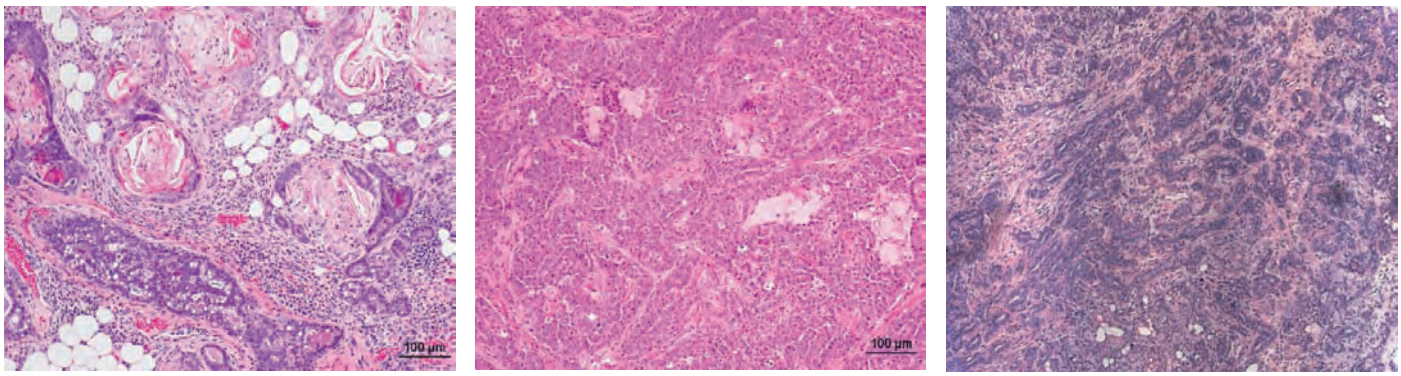


Appendix 1: Abnormal Mammary Gland Development in CUX1 Transgenic Mice
Mammary gland whole-mounts from p75, p110 and p200 CUX1 mice at 5 weeks (A) and 3 months (B) were stained with hematoxylin. In p75 mice, the mammary gland has progressed much beyond the lymph node at 5 weeks (A). Arrows represent elongation distance from the lymph node. In p200 mice, no difference was noted at 5 weeks, but more extensive side-branching and more terminal end-buds were observed at 3 months (B). 1300 CUX1 (C) and PCNA (D) immunohistochemistry was performed on wild-type and transgenic mammary glands.

	Wild-type n=74	p75 n=30	p110 n=87	p200 n=51
Total Number with Tumors	14 (18.9%)	**12 (40%)***	**43 (49.4%)*****	**23 (45%)****
Histiocytic sarcoma (uterus)	7 (9.5%)	1 (3.3%)	11 (12.6%)	2 (3.9%)
Mammary Gland Tumors	3 (4.1%)	**6 (20%)***	**9 (10.3%)**	**10 (19.6%) ****
Hematopoietic	0	0	3 (3.4)	1 (2.0%)
Lung Tumors	6 (8.1%)	**8 (26.7%)***	**18 (20.7%)***	**9 (17.6)***
Other	2 (2.7%)	0	6 (6.9%)	1 (2.0%)

Appendix 2: Percentages and types of tumors developed in the different mouse lines of pure FVB backgrounds.
* p-value ≤ 0.05, ** p-value ≤ 0.01, *** p-value ≤ 0.001

A

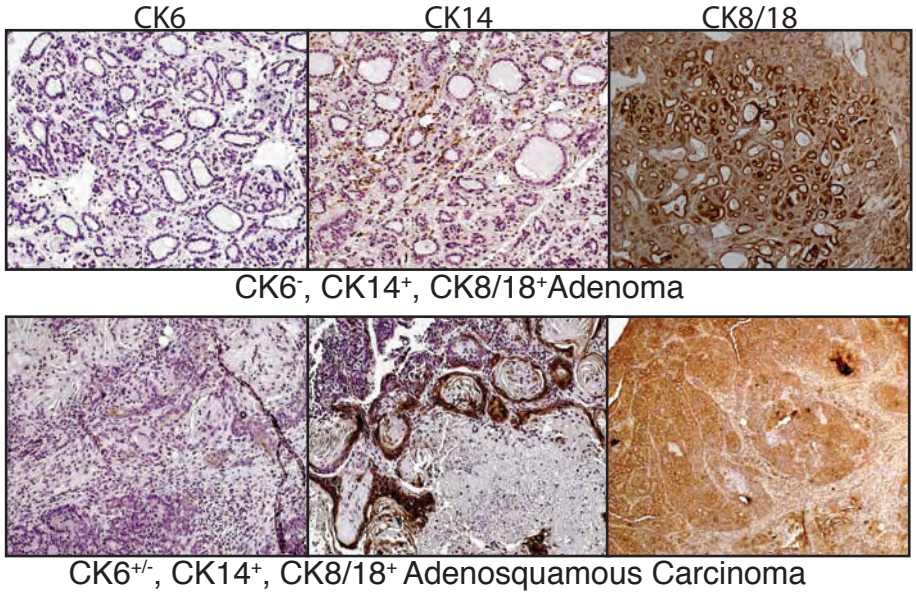


adenosquamous carcinoma | solid carcinoma | adenomyoepithelioma

B

Type of mammary lesion	WT	p75	p110	p200
adenosquamous	33%	70%	20%	70%
adenoma: tubular acinar	0%	0%	10%	0%
adenomyoepithelioma	0%	10%	10%	0%
solid carcinoma	67%	10%	50%	10%
Undiagnosed	0%	10%	10%	20%

C



$CK6^{-}$, $CK14^{+}$, $CK8/18^{+}$Adenoma

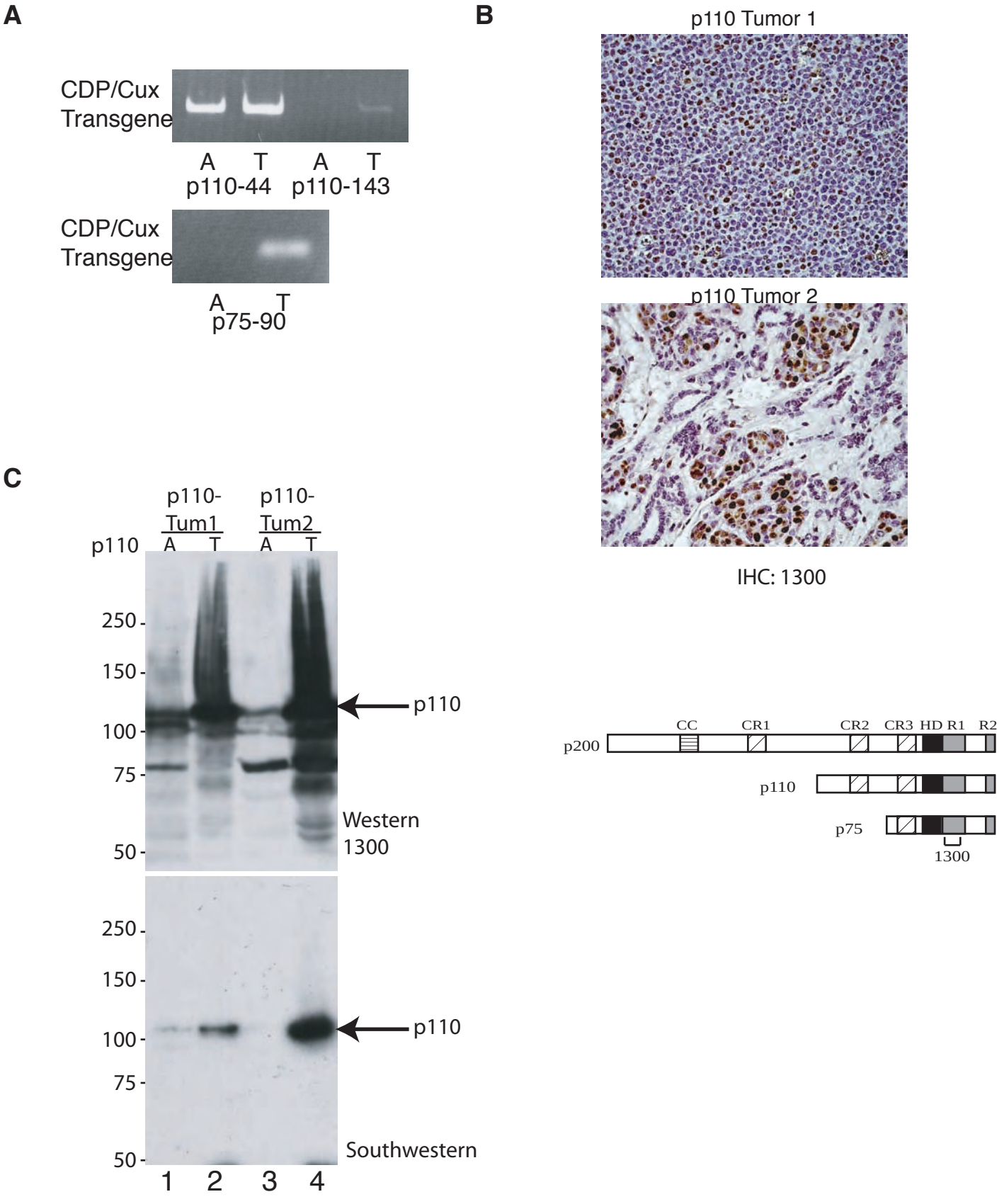
$CK6^{+/-}$, $CK14^{+}$, $CK8/18^{+}$ Adenosquamous Carcinoma

Appendix 3: Mammary gland tumors are heterogeneous

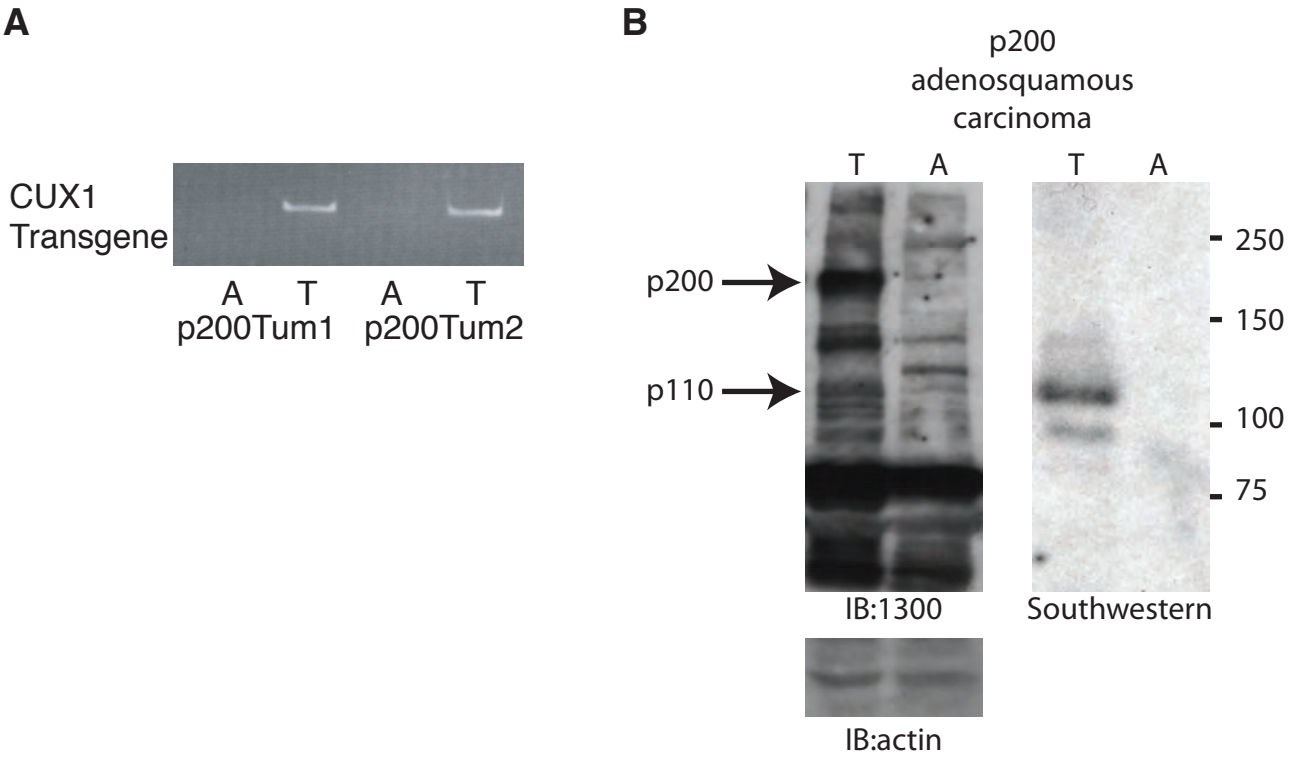
(A)H&E stainings showing the different types of tumors seen in the CUX1 transgenic mice

(B)Percentages and types of tumors developed in the different mouse lines

(C)Mammary tumors from CUX1 transgenic mice express markers of progenitor cells (CK6), myoepithelial cells (CK14) and luminal epithelial cells (CK8/18)



Appendix 4: The transgene is expressed and active in the tumors.
(A) RT-PCR analysis shows specific transgene expression at the mRNA level in the tumors.
(B) Immunohistochemistry using the 1300 CUX1 antibody on p110 tumors
(C) Western blot and southwestern blot analyses on adjacent (A) and tumor (T, Tum) tissue from transgenic mice.

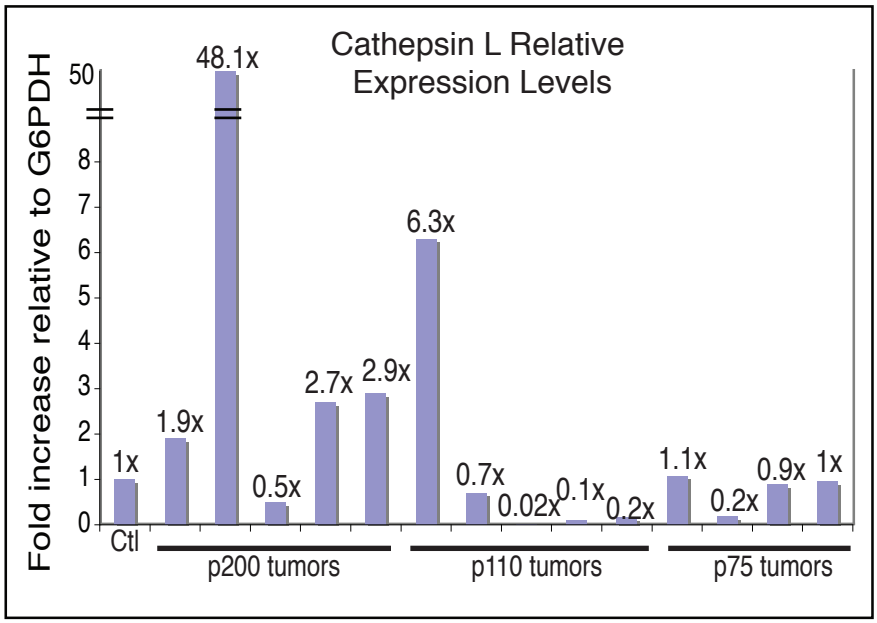


Appendix 5: Increased p110 expression and activity in p200 tumors

(A) RT-PCR analysis shows specific transgene expression at the mRNA level in the tumors.

(B) Western blot and southwestern blot analyses on adjacent (A) and tumor (T, Tum) tissue from p200 transgenic mice.

A

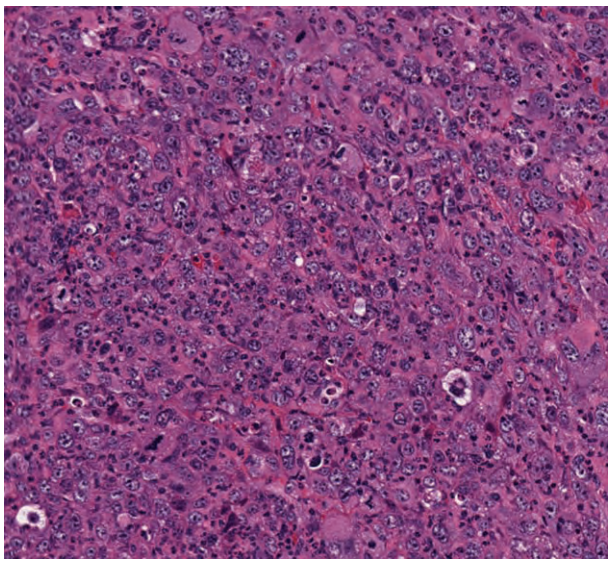


B

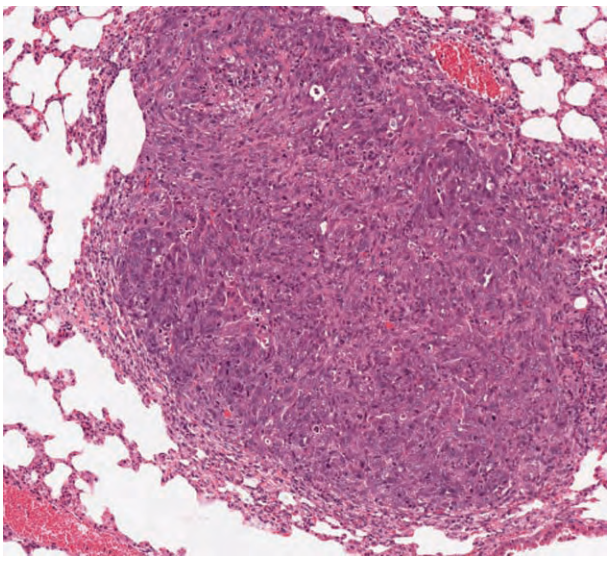
```
                                    a.a.59    a.a.61
K-Ras     GATATTCTCGACACA GCAGGT CAAGAGGAGTACA

tumor1    GATATTCTCGACACA GCAGGT CAAGAGGAGTACA
tumor2    GATATTCTCGACACA GCAGGT CTAGAGGAGTACA  ← Mutated
tumor3    GATATTCTCGACACA GCAGGT CAAGAGGAGTACA
```

Appendix 6: Increased cathepsin L expression in p200 tumors
(A) Cathepsin L expression in p200, p110 and p75 mammary gland tumors. To obtain fold increase, every tumor was compared to its adjacent mammary gland.
(B) One p200 tumor out of 3 analyzed contained an activating K-ras mutation (Q61L)

A

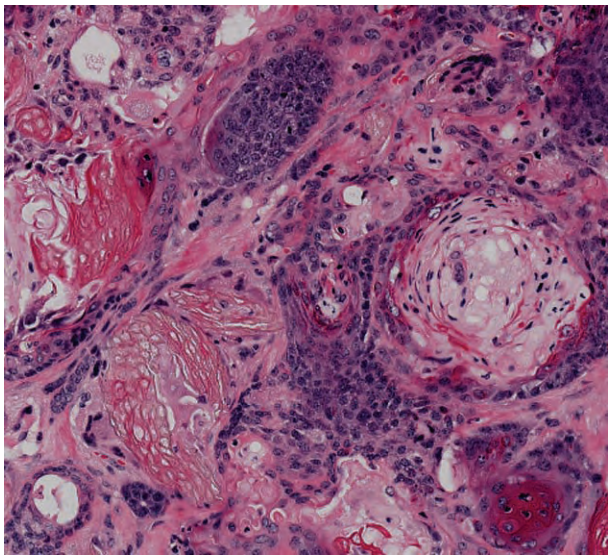


p75 mammary gland tumor

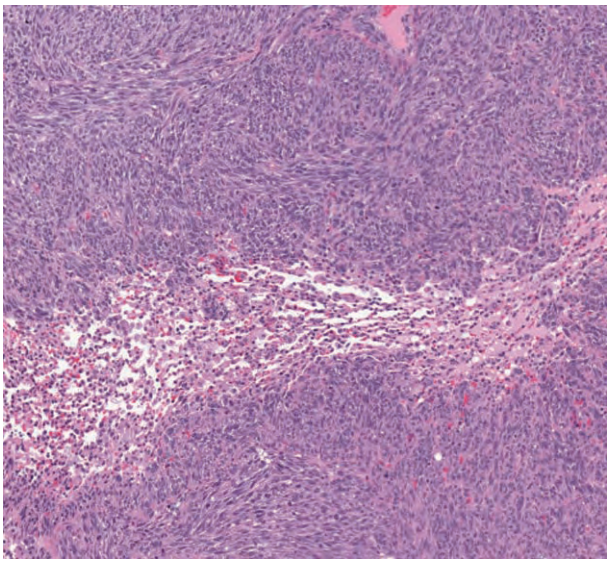


metastasis to lungs

B

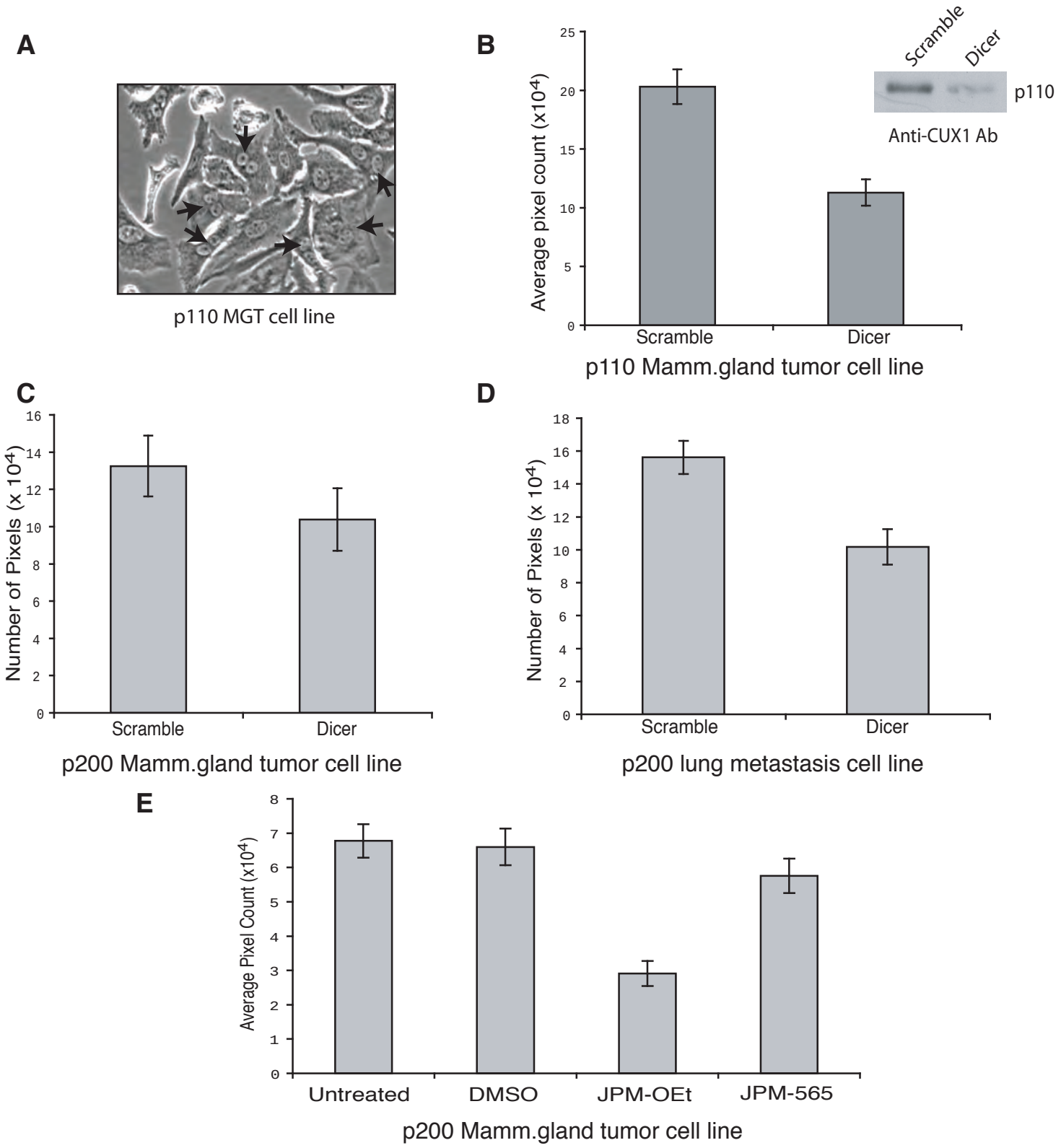


p200 mammary gland tumor

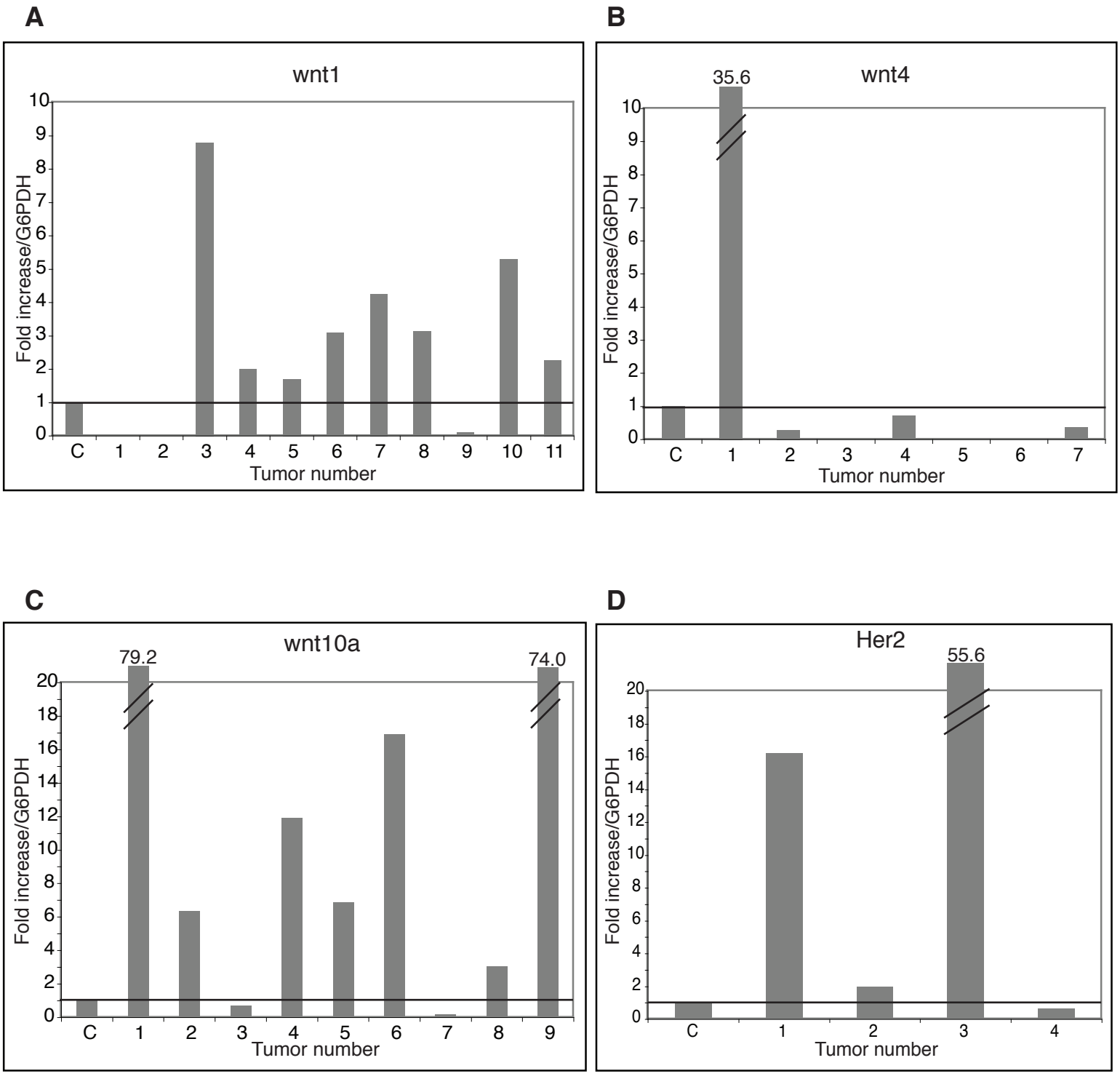


metastasis to lungs

Appendix 7: Some CUX1 mammary gland tumors metastasized to lungs
Some mammary gland tumors metastasized to lungs in p75 transgenic (A) and p200 transgenic (B) mice.



Appendix 8: Knock-down of exogenous CUX1 in tumor-derived cell lines results in reduced motility
(A) Picture of p110-derived cell line with two nuclei per cell.
Migration assay using p110-mammary gland tumor-derived cell lines(B), p200-mammary gland tumor-derived cell lines (C), and in p200-lung metastasis-derived cell line (D).
(E) Migration assay using p200-mammary gland tumor-derived cell line treated with a permeable (JPM-OEt) or non-cell-permeable (JPM-565) cysteine protease inhibitor.



Appendix 9: Expression of wnt genes and Her2 in CUX1 tumors

cDNA was prepared from the tumor samples and from their corresponding adjacent mammary glands and Real-Time PCRs were performed to look at expression of wnt1 (A), wnt 4 (B), wnt10a (C) and Her2 (D). To obtain fold increase, expression of each gene in the tumor was compared to its expression in the corresponding adjacent mammary gland and normalized over G6PDH. c=control mouse

Appendix 10

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CDP/Cux Transgenic Mice: The role of CDP/Cux in Cancer
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Abstract

The CUX1 transcription factor is involved in several processes including cell cycle progression, cell migration and invasion and the determination of cell-type identity in several tissues. The full-length protein of 200 kDa (p200) is proteolytically processed by nuclear cathepsin L at the G1/S transition into an isoform of 110 kDa (p110). A second isoform of 75 kDa (p75) is generated from an alternative mRNA. The p110 and p75 isoforms are overexpressed in different types of cancers, such as in leiomyomas and breast cancers. In tissue culture, p110 stimulates cell proliferation by accelerating the G1/S transition.

To investigate the oncogenic potential of CUX1, we engineered transgenic mice overexpressing p75, p110 or p200 CUX1 under the control of the mouse mammary tumor virus promoter (MMTV). Each transgene was specifically integrated into the hprt locus and then backcrossed into the FVB and the C57BL6 genetic backgrounds. In a mixed background p75 CUX1 mice developed a myeloproliferative disease-like myeloid leukemia. In the FVB background, each CUX1 isoform caused an abnormal development of the mammary gland and, after a long latency and with a low penetrance, cancer in the mammary gland, the uterus and the lungs. Mammary gland tumors in the three lines are very heterogeneous, with some being solid carcinomas, others containing areas of squamous metaplasia or papillary differentiation and others being more glandular. Some contain different histological types within the same mass. These results suggest that CUX1 causes the malignant transformation of a precursor cell, which can then give rise to different lineages, implying that the tumors developed in these mice are of basal origin.

Overall, these results confirm the oncogenic potential of CUX1 in several tissues and cell types and reveal a particular tropism of the p75 isoform towards specific cell types within the hematopoietic system. In contrast, in the mammary gland the 3 CUX1 isoforms appear to target the same precursor cell.